Australian Government

Patent Office
Canberra

I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2003901267 for a patent by MONASH UNIVERSITY as filed on 19 March 2003.

COMMONWEALTH OF AUSTRALIA
AUSTRALIA
PATENT OFFICE

WITNESS my hand this
Fifth day of April 2004

J. Billingsley

JULIE BILLINGSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

P/00/009
Regulation 3.2

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

APPLICANT: **MONASH UNIVERSITY**

INVENTION TITLE: **ASSESSMENT METHOD**

The invention is described in the following statement:

ASSESSMENT METHOD

Technical Field

The present invention relates to the use of activin and follistatin to predict outcomes in acute systemic inflammatory conditions. Measurement of the concentration of activin and/or follistatin in blood or its derivatives or other tissue fluids predicts the outcome of the condition. The invention provides a simple test to predict the outcome of an acute inflammatory episode. In the case of sepsis the test will show if a patient is likely to survive and to therefore identify those patients in need of more aggressive treatment.

Background Art

Sepsis is a major cause of morbidity and mortality worldwide and is the leading non-coronary cause of death in intensive care units. More than 700,000 cases of severe sepsis occur in the US annually at a healthcare cost of $17 billion annually.

Intense interest has focussed on the ability to discriminate between those patients that die from sepsis and those that will survive. A number of diagnostic tests including body temperature, leukocyte count and various blood markers such as C-reactive protein, procalcitonin and various cytokines have been evaluated. While a number of these show predictive value in discriminating patient outcome, there is a need to continue to evaluate new markers or combinations of markers to improve diagnostic accuracy.

Activin and follistatin are protein factors that are present at low levels in blood. They have only been recently associated with inflammatory diseases.

The above discussion of background art is included to explain the context of the present invention. It is not to be taken as an admission that any of the documents or other material referred to was published, known or part of the common general knowledge in Australia at the priority date of any one of the claims of this specification.

Description of the Invention

Throughout the description and claims of this specification, the word "comprise" and variations of that word, such as "comprising" and "comprises" are not intended to exclude other additives, steps or integers.

In sheep models of acute inflammatory challenge, activin and follistatin are elevated in the blood. However, until now there has been no human data to suggest

that activin or follistatin are useful predictors of clinically important inflammatory diseases such as sepsis. While some workers have looked at levels of for instance, follistatin, in inflammatory conditions there has been no recognition until now that examination of those levels can provide useful information of the management of patients with acute inflammatory conditions. Thus for instance Michel et al (13) showed that follistatin is elevated in septicemia but did not find a useful correlation with outcome/prognosis. Michel et al (14) showed that follistatin is elevated in meningitis but did not find that this was correlated directly with clinical outcome. Phillips et al (15) contains a limited data set from food poisoning patients. Again a correlation with outcome/prognosis for patients with acute inflammatory conditions is not suggested.

Our data shows that high levels of activin and/or follistatin are associated with a poor outcome (patient death) for patients with acute inflammatory conditions, whereas lower levels correlate with patient survival.

The present invention improves diagnostic accuracy (patient outcome).

The principal application or use for the invention is prediction of patient outcome in patients hospitalised with sepsis.

The invention also has application in the assessment of patient response in other acute inflammatory diseases such as meningitis and appendicitis or traumatic injuries such as surgery and burns.

In a first aspect the present invention provides a method for predicting the outcome of an acute inflammatory or traumatic condition in a patient, which method comprises measuring the level of activin and/or follistatin in the patient.

Typically the method involves measuring the level of activin and/or follistatin in a sample taken from the patient.

The sample may be a blood sample, a serum sample or a sample from another body fluid or tissue which shows variation in activin and/or follistatin levels correlating with the presence and extent of an acute inflammatory or traumatic condition in the patient.

The sample may be analysed in the form in which it is taken from the patient or alternatively it may be further processed prior to analysis.

Measuring of the levels of activin and/or follistatin may be performed by various immunological means, including radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), western blot, ELISPOT, binding assays where the binding partner may be established binding proteins or fragments thereof for either activin and follistatin such as α2-macroglobulin, myostatin, follistatin-related protein

(follistatin-3), binding assays incorporating activin receptor fragments or other cellular binding proteins, or cell culture systems where the activity of activin and/or follistatin is assessed by measuring a cellular endpoint such as proliferation, protein production or other means.

In one embodiment of the present invention the method relies on the measurement of only activin and/or follistatin.

In another embodiment of the present invention the method relies on the measurement of activin and/or follistatin in combination with measurement of existing markers.

The inflammatory conditions and/or traumatic conditions which can be assessed by the method of the invention include sepsis, meningitis, burns, surgery and appendicitis.

In the case of sepsis the method of the invention permits determination of the likely outcome of the disease. For patients assessed as having a poor prognosis, more aggressive treatments of the condition can be initiated.

For a patient with sepsis a level of activin and/or follistatin at least about 2 times higher than levels within the normal range for the method of assay by which the levels are measured is indicative of poor prognosis.

More particularly for a patient with sepsis a level of activin and/or follistatin at least about 3 times higher than levels within the normal range for the method of assay by which the levels are measured is indicative of poor prognosis.

In one embodiment of the invention the level of activin and/or follistatin functions as a predictor of possible death when the activin level is greater than 0.3ng/ml over a 24 hour period and/or the level of follistatin is greater than 20ng/ml over a 24 hour period when measured by assays as herein described.

The combination of level with time period overcomes difficulty associated with the fact that patients being assessed will be at different stages of disease.

In a second aspect the present invention provides the use of activin and/or follistatin in the manufacture of a kit for use in a method for determining the severity of an inflammatory or traumatic condition in a patient, which method comprises measuring the level of activin and/or follistatin in the patient.

In a third aspect the present invention provides a kit for use in a method for determining the severity of an inflammatory or traumatic condition in a patient, which method comprises measuring the level of activin and/or follistatin in the patient, the kit comprising means for measuring the level of activin and/or follistatin in the patient.

In a fourth aspect the present invention provides a method of treating a patient suffering an acute inflammatory or traumatic condition which method comprises determining the severity of the inflammatory or traumatic condition in the patient by measuring the level of activin and/or follistatin in the patient and based on the level of activin and/or follistatin measured applying a suitably aggressive treatment of the inflammatory or traumatic condition.

It is to be appreciated that alterations and/or additions may be made to the parts and/or embodiments previously described without departing from the spirit or ambit of the invention.

Levels of activin in normal patient sera have been measured using a specific 2 site EIA as reported by Loria P. *et al* in European Journal of Endocrinology (1998) 139 487-492.The results they reported were as follows:

Table 1 Serum concentrations of activin A in patients .

	Women		Men			Total	
Age group (years)	n	Activin A (ng/ml)	n	Activin A (ng/ml)	p	n	Activin A (ng/ml)
20–30	18	0.50±0.17	10	0.5260.10	NS	28	0.5160.15
30–40	20	0.55 ±0.28	26	0.63 ±0.10	NS	46	0.60 ±0.20
40–50	50	0.77±0.21	25	0.75±0.14	NS	75	0.77±0.19
50–60	31	0.58±0.13	15	1.05±0.17	<0.001	46	0.73±0.27
60–70	12	0.59±0.10	14	1.11±0.20	<0.001	26	0.87±0.31
70–90	20	0.67±0.10	16	1.09±0.18	<0.001	36	0.86±0.26
Total	151	0.64±0.21	106	0.84±0.26	<0.001	257	0.73±0.25

Data are expressed as means ±S.D. n, number of patients studied. Statistical analysis was performed by Student's t-test for unpaired data compared with the same age group of different sex.

While these authors used the same activin ELISA as used in the examples below, they used a different reference standard. To compare the two sets of results (theirs and ours) you need to apply a correction factor of ~2.4, that is their values are ~2.4 times higher than what we would measure. This is an important point in defining normal ranges and concentrations: these will vary from assay to assay. While the inventors have assigned numeric cutoffs with the assays they used that indicate poor prognosis the skilled addressee will recognise that different assays have different numeric cutoff values.

For follistatin the normal range is also variable depending on the assay. A typical normal range would be <12 ng/ml or more conservatively <15 ng/ml.

1. Data from normal patients presented in the examples below: "normal" controls were all <12 ng/ml.

2. Figure 2 Phillips et al (16) gives a normal range for follistatin as <10 ng/ml.

3. Figure 3 from Phillips et al (17) reports pretreatment levels of follistatin in patients with no inflammatory syndromes as 10-15 ng/ml.

4. Figure 2 from Reame et al (18) shows in young and middle aged women normal levels of follistatin are <10 ng/ml.

Brief Description of the Accompanying Drawings

Figure 1 shows a time course of activin, FS and C-reactive protein (CRP) concentrations in serum of patients with septicemia. Patient numbers correspond to the numbers in Table 2. In each diagram on the X-axis, the time points of blood sampling are shown (first sample taken at 0 h). On the left Y-axis, FS serum concentrations in ng/ml and on the right Y-axis activin serum concentrations in ng/ml are shown. The second Y-axis on the left is the scale for the CRP serum concentrations in mg/l.

Figure 2 shows a time course of activin, FS and C-reactive protein (CRP) concentrations in serum of patient A.S. a 31 year old male diagnosed with *Neisseria meningitidis* meningitis and sepsis who subsequently died.

Figure 3 shows a time course of activin, FS and C-reactive protein (CRP) concentrations in serum of patient M.B. a 43 year old female diagnosed with gastroenteritis who was cured.

Figure 4 shows a time course of activin, FS and C-reactive protein (CRP) concentrations in serum of patient Ho a 48 year old male diagnosed with a cutaneous infection. It is believed the patient recovered.

Figure 5 shows a time course of activin, FS and C-reactive protein (CRP) concentrations in serum of patient M.F. a 29 year old female diagnosed with *Staphylococcus aureus* sepsis who was cured.

Figure 6 shows a time course of activin, FS and C-reactive protein (CRP) concentrations in serum of patient M.M. a 52 year old male diagnosed with cirrhosis who subsequently died.

Figure 7 shows a time course of activin, FS and C-reactive protein (CRP) concentrations in serum of patient U.D. a 33 year old male diagnosed with *Streptococcus pneumoniae* sepsis who subsequently died.

Figure 8 shows a time course of activin, FS and C-reactive protein (CRP) concentrations in serum of patient W.L. an 87 year old male diagnosed with pneumonia who subsequently died.

Figure 9 shows a time course of activin, FS and C-reactive protein (CRP) concentrations in serum of patient W.S. a 58 year old male diagnosed with intracranial bleeding. The outcome for this patient is unknown.

Best and Other Methods of Carrying Out the Invention

EXAMPLE 1

Materials and Methods

As part of their routine clinical management, serial blood samples were collected from seven female and eight male patients of different age who suffered from septic infections of different grades of severity. After completion of the clinical routine analyses, follistatin and activin levels were measured in the remnants of serum samples. The samples were stored frozen at -20 °C until assaying. Since activin and follistatin serum levels increase with age (1,2), serum samples from age- and sex-matched healthy volunteers served as controls. All samples from diseased and healthy subjects were treated in the same way.

Patients were categorized for septicemia according to the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM) Consensus criteria (manifestation of two or more of the following clinical conditions: body temperature > 38 °C or < 36 °C; heart rate > 90 beats/minute; respiratory rate > 20 breaths/minute or $PaCO_2$ < 32 mmHg; white blood cell count > 12000cells/mm^3, < 4000 cells/mm^3, or > 10 % immature forms). For twelve of the fifteen patients, the diagnosis of septicemia was confirmed by culture of the infectious organism from blood. In three cases the culture of the infectious organism failed due to the rapid implementation of antibiotic treatment.

Activin A concentrations in serum were measured using a specific ELISA which detects both follistatin-bound and free activin (3), with the following

modifications. The standard used was human recombinant activin A. The assay sensitivity was 0.1 ng/ml and the intra- and inter-assay coefficients of variation were 4.7% and 7.8% respectively. Serum samples were assayed against the standard diluted in 5 % bovine serum albumin (BSA) in phosphate-buffered saline (PBS, 0.01 M). Follistatin concentrations in serum were measured with a radioimmunoassay validated for human follistatin as previously described (4). The standard employed was human recombinant follistatin 288, but the assay crossreacts with hrFS 315 (35.9%). The assay sensitivity was 2.0ng/ml and the intra- and interassay coefficients of variation were both<4.9%. The assay measures total follistatin (free and bound). Numbers of leukocytes, serum creatinine levels, and serum CRP levels were determined by clinical routine methods in the department of Clinical chemistry of the University of Göttingen.

Differences in activin and follistatin serum concentrations between septic patients and matched healthy volunteers were analysed by paired t-test. Correlations between measured parameters were calculated with Pearson correlation. Analyses were performed using Graph Pad Prism 3.0 (San Diego, CA, USA.

Results and Discussion

Peak activin and follistatin serum concentrations of patients with septicemia were elevated compared with concentrations in age- and sex-matched controls (Table 2). The median of the maximum activin concentration was 3.9-fold higher than the median in healthy controls ($p < 0.01$); the median of the maximum follistatin concentrations of septicaemic patients was 2.6-fold higher than the median of the follistatin concentrations in healthy persons ($p < 0.01$). The magnitude of the activin and follistatin increase during septicemia varied among individuals. Figure 1 depicts the individual profiles of serum activin and follistatin in the seven female (A) and

eight male (B) patients and the corresponding serum levels of C-reactive protein (CRP), an indicator of inflammation. Most of the diagrams in Figure 1 show that activin and follistatin serum levels track each other and follow changes in CRP levels. Overall, activin and follistatin concentrations were correlated with each other (r^2 = 0.64). There was no apparent relation of activin and follistatin serum concentrations to the number of leukocytes (r^2 =0.09). The parallel profiles of follistatin, activin, and CRP suggest a causal relationship between bacterial infection and elevated activin and follistatin serum levels in follistatin. The observed increases in follistatin and activin serum concentrations during the inflammatory response are in accordance with observations in animal experiments, where IL-1β or LPS injections caused significantly elevated follistatin and activin serum levels (5, 6, 7). The onset of septicaemia is often unnoticed, whereas in most cases blood sampling started with obvious signs of the disease; this made it impossible to determine whether the activin, follistatin or CRP serum concentration was the first to rise at the beginning of the infection.

The normal creatinine serum concentrations in most patients and the molecular size of the glycosylated follistatin (39-45 kDa) and activin (25kDa) molecules make an altered renal function as the sole cause of increased serum concentrations in sepsis unlikely. The tissues or cell types which contribute to the activin and follistatin serum levels in normal and infected animals or humans are currently unknown. Leukocyte counts did not show a strong correlation to follistatin and activin levels in septicemic patients (r^2 = 0.09) and therefore leukocytes may not be the primary source of increased follistatin and activin levels during septicemia. Nevertheless, the lack of correlation with total leukocyte counts does not rule out that specific populations of cells preferentially release activin and/or follistatin, as has been documented for human monocytes in response to LPS.

Well-balanced steady-state levels of follistatin and activin govern the growth, differentiation and behaviour of many cell types, and pharmacological doses of follistatin and activin affect pituitary hormone secretion in animals. Therefore it is conceivable that elevated follistatin and activin serum levels can also influence endocrine hormone patterns or paracrine interactions in tissues. Elevated serum concentrations of activin and/or follistatin have been reported in a number of pathological states, including hypertension during pregnancy, renal failure, liver dysfunction and various carcinomas (8-12). Nevertheless, the role of these proteins in the disease process is poorly defined. The need for a well-balanced equilibrium of follistatin and activin could be one explanation for the tight correlation of both factors in serum during sepsis (r^2 =0.64). In terms of inflammatory processes, emerging data suggest activin can have both pro- and anti-inflammatory effects on cell responses and cytokine production, which may account for its the elevated levels in patients with septicemia. Nevertheless, these studies have focussed largely on cell cultures of primary immune cells or cell lines. Therefore the pathophysiological relevance of activin and follistatin release to septicemia remains to be elucidated.

Table 2

Number	Diagnosis	Age	Sex	Septicemia (ng/ml) Activin/Follistatin		Control (ng/ml) Activin/Follistatin		Outcome
1	Sepsis (Staph. aureus)	64	f	0.42	25.77	0.15	4.84	Deceased
2	Sepsis & meningitis (Strept. pneu.)	72	f	0.65	14.48	0.18	10.4	Cured
3	Sepsis & meningitis (Neisseria meningitidis)	42	f	0.15	5.27	0.07	5.7	Cured
4	Sepsis & meningitis (Strept. pneu.)	56	f	0.59	15.00	0.1	5.6	Deceased
5	Sepsis & encephalitis & endocarditis (coagulase-negative Staph.)	84	f	1.97	39.64	0.18	10.4	Deceased *
6	Sepsis (E. coli)	67	f	0.61	42.28	0.16	5.7	Deceased
7	Sepsis (Neisseria meningitidis)	33	f	2.31	76.14	0.08	5.1	Deceased
8	Sepsis	66	m	0.62	16.82	0.13	3.88	Deceased
9	Sepsis & meningitis (Strep. pneu.)	37		0.19	16.39	0.11	5.75	Cured
10	Sepsis (Staph. aureus)	70	m	0.81	21.9	0.15	6.48	Deceased
11	Sepsis & meninigtis (Neisseria meningitidis)	46	m	0.18	5.26	0.13	5.34	Cured
12	Sepsis (coagulase-negative Staph.)	75	m	0.19	15.01	0.18	11.3	cured
13	Sepsis	62	m	0.43	9.97	0.17	10.84	deceased
14	Sepsis & pneumonia	76	m	0.28	12.47	0.18	9.36	cured
15	Sepsis (Staph. Epidermidis)	71	m	0.28	14.88	0.17	7.71	cured
	Median			0.59	15	0.15	5.75	

Table 2 shows a comparison of activin and FS serum levels of patients with septicemia and healthy age and sex-matched controls. The concentration given for septic patients is the peak level observed across multiple samples. Staph., Staphylococcus; Strept., Streptococcus; pneu., pneumoniae; m, male; f, female; control versus septicemia: $p = 0.004$ for activin concentrations and $p = 0.009$ for FS concentrations (paired Wilcoxon rank test). *patient died soon after discharge from clinic.

With the exception of one patient, in all deceased cases there is a "significant" increase in follistatin in sepsis compared to controls (increase in follistatin is normally associated also with an increase in activin). Conversely, where the increase in follistatin is less than approximately two-fold compared with controls, an association with a positive outcome can be detected.

EXAMPLE 2

Follistatin and activin levels were assessed over time in a further 8 patients as follows.

The methodology is exactly the same as used for Example 1.

Figures 2 to 9 show the levels of activin, follistatin and CRP in these patients.

Figure 2 shows a time course of activin, FS and C-reactive protein (CRP) concentrations in serum of patient A.S. a 31 year old male diagnosed with *Neisseria meningitidis* meningitis and sepsis who subsequently died. For this patient serum activin levels ranged between 0.100 and 0.150ng/ml while follistatin levels ranged between 9 and 10ng/ml.

Figure 3 shows a time course of activin, FS and C-reactive protein (CRP) concentrations in serum of patient M.B. a 43 year old female diagnosed with gastroenteritis who was cured. For this patient serum activin levels ranged between 0.100 and 0.150ng/ml while follistatin levels ranged between 2 and 4ng/ml .

Figure 4 shows a time course of activin, FS and C-reactive protein (CRP) concentrations in serum of patient Ho a 48 year old male diagnosed with a cutaneous infection. It is believed the patient recovered. For this patient serum activin levels ranged between 0 and 0.2ng/ml while follistatin levels ranged between 9 and 25ng/ml.

Figure 5 shows a time course of activin, FS and C-reactive protein (CRP) concentrations in serum of patient M.F. a 29 year old female diagnosed with *Staphylococcus aureus* sepsis who was cured. For this patient serum activin levels ranged between 0.060 and 0.105ng/ml while follistatin levels ranged between 9 and 15ng/ml.

Figure 6 shows a time course of activin, FS and C-reactive protein (CRP) concentrations in serum of patient M.M. a 52 year old male diagnosed with cirrhosis who subsequently died. For this patient serum activin levels ranged between 0.25 and 0.50ng/ml while follistatin levels ranged between 5 and 32ng/ml.

Figure 7 shows a time course of activin, FS and C-reactive protein (CRP) concentrations in serum of patient U.D. a 33 year old male diagnosed with *Streptococcus pneumoniae* sepsis who subsequently died. For this patient serum activin levels ranged between 0 and 0.68ng/ml while follistatin levels ranged between 5 and 125ng/ml.

Figure 8 shows a time course of activin, FS and C-reactive protein (CRP) concentrations in serum of patient W.L. an 87 year old male diagnosed with pneumonia who subsequently died. For this patient serum activin levels ranged between 0.11 and 0.33ng/ml while follistatin levels ranged between 17 and 32ng/ml.

Figure 9 shows a time course of activin, FS and C-reactive protein (CRP) concentrations in serum of patient W.S. a 58 year old male diagnosed with intracranial bleeding. The outcome for this patient is unknown. For this patient serum activin levels ranged between 0.07 and 0.15ng/ml while follistatin levels ranged between 2.5 and 7.5ng/ml.

Typically patients in whom activin levels remained below 0.3 ng/ml and follistatin levels remained below 20 ng/ml recovered whereas patients with higher levels died.

EXAMPLE 3

Follistatin and activin levels were assessed over time in a further group of patients with meningitis and other brain disorders.

The methodology was as for Example 1. For measuring activin A in CSF samples, the standard diluent used was 0.05 % BSA in PBS to match the protein concentration in the samples. A 20% solution of BSA in PBS (25 µL) was added to the wells before the addition of CSF samples as this was found to enhance the reproducibility of the assay.

References

1. Wakatsuki M, Shintani Y, Abe M, Liu ZH, Shitsukawa K & Saito S Immunoradiometric assay for follistatin: serum immunoreactive follistatin levels in normal adults and pregnant women. *J Clin Endocrinol Metab* 1996 **81** 630-634.

2. Loria P, Petraglia F, Concari M, Bertolotti M, Martella P, Luisi S, *et al.* Influence of age and sex on serum concentrations of total dimeric activin A. *Eur J Endocrinol* 1998 **139** 487-492.

3. Knight PG, Muttukrishna S & Groome NP Development and application of a two-site enzyme immunoassay for the determination of 'total' activin-A concentrations in serum and follicular fluid. *J Endocrinol* 1996 **148** 267-279.

4. O'Connor AE, McFarlane JR, Hayward S, Yohkaichiya T, Groome NP & de Kretser DM Serum activin A and follistatin concentrations during human pregnancy: a cross-sectional and longitudinal study. *Hum Reprod* 1999 **14** 827-832.

5. Jones KL, Brauman JN, Groome NP, de Kretser DM & Phillips DJ Activin A release into the circulation is an early event in systemic inflammation and precedes the release of follistatin. *Endocrinology* 2000 **141** 1905-1908.

6. Klein R, Clarke IJ, Hedger MP & Robertson DM Plasma follistatin concentrations increase following lipopolysaccharide administration in sheep. *Clin Exp Pharmacol Physiol* 1996 **23** 754-755.

7. Phillips DJ, Hedger MP, McFarlane JR, Klein R, Clarke IJ, Tilbrook AJ, *et al.* Follistatin concentrations in male sheep increase following sham castration/castration or injection of interleukin-1 beta. *J Endocrinol* 1996 **151** 119-124.

8. Sakamoto Y, Shintani Y, Harada K, Abe M, Shitsukawa K & Saito S Determination of free follistatin levels in sera of normal subjects and patients with various diseases. *Eur J Endocrinol* 1996 **135** 345-351.

9. Harada K, Shintani Y, Sakamoto Y, Wakatsuki M, Shitsukawa K & Saito S Serum immunoreactive activin A levels in normal subjects and patients with various diseases. *J Clin Endocrinol Metab* 1996 **81** 2125-2130.

10. Pirisi M, Fabris C, Luisi S, Santuz M, Toniutto P, Vitulli D, *et al.* Evaluation of circulating activin-A as a serum marker of hepatocellular carcinoma. *Cancer Detect Prev* 2000 **24** 150-155 .

11. Patella S, Phillips DJ, de Kretser DM, Evans LW, Groome NP & Sievert W Characterisation of serum activin-A and follistatin and their relation to virological and histological determinants in chronic viral hepatitis. *J Hepatol* 2001 **34** 576-583.

12. Yuen M-F, Norris S, Evans LW, Langley PG & Hughes RD Transforming growth factor-beta 1, activin and follistatin in patients with hepatocellular carcinoma and patients with alcoholic cirrhosis. *Scand J Gastroenterol* 2002 **37** 233-238.

13. Michel U, Shintani Y & Nau R. Serum follistatin concentrations are increased in patients with septicaemia. *Clin Endocrinol* 1998 **48** 413-417.

14. Michel U, Ebert S, Schneider O, Shintani Y, Bunkowski S, Smirnov A, Stringaris AK, Gerber J, Bruck W & Nau R. Follistatin (FS) in human cerebrospinal fluid and regulation of FS expression in a mouse model of meningitis. Eur J Endocrinol 2000 143 809-816.

15. Phillips DJ, Jones KL, Scheerlinck J-PY, Hedger MP & de Kretser DM. Evidence for activin A and follistatin involvement in the systemic inflammatory response. Mol Cell Endocrinol 2001 180 155-162.

16. Phillips (16) DJ, Jones KL, Scheerlinck J-PY, Hedger MP & de Kretser DM. Evidence for activin A and follistatin involvement in the systemic inflammatory response. Mol Cell Endocrinol 2001 180 155-162.

17. Phillips (17) DJ, Jones KL, McGaw DJ, Groome NP, Smolich JJ, Parsson H & de Kretser DM. Release of activin and follistatin during cardiovascular procedures is largely due to heparin administration. J Clin Endocrinol Metab 2000 85 2411-2415.

18. Reame(NE, Wyman TL, Phillips DJ, de Kretser DM & Padmanabhan V. Net increase in stimulatory input resulting from a decrease in inhibin B and an increase in activin A may contribute in part to the rise in follicular phase follicle-stimulating hormone of aging cycling women. J Clin Endocrinol Metab 1998 83 3302-3307

DATED: [19 MARCH 2003]

PHIILLIPS ORMONDE & FITZPATRICK

Attorneys for **MONASH UNIVERSITY**

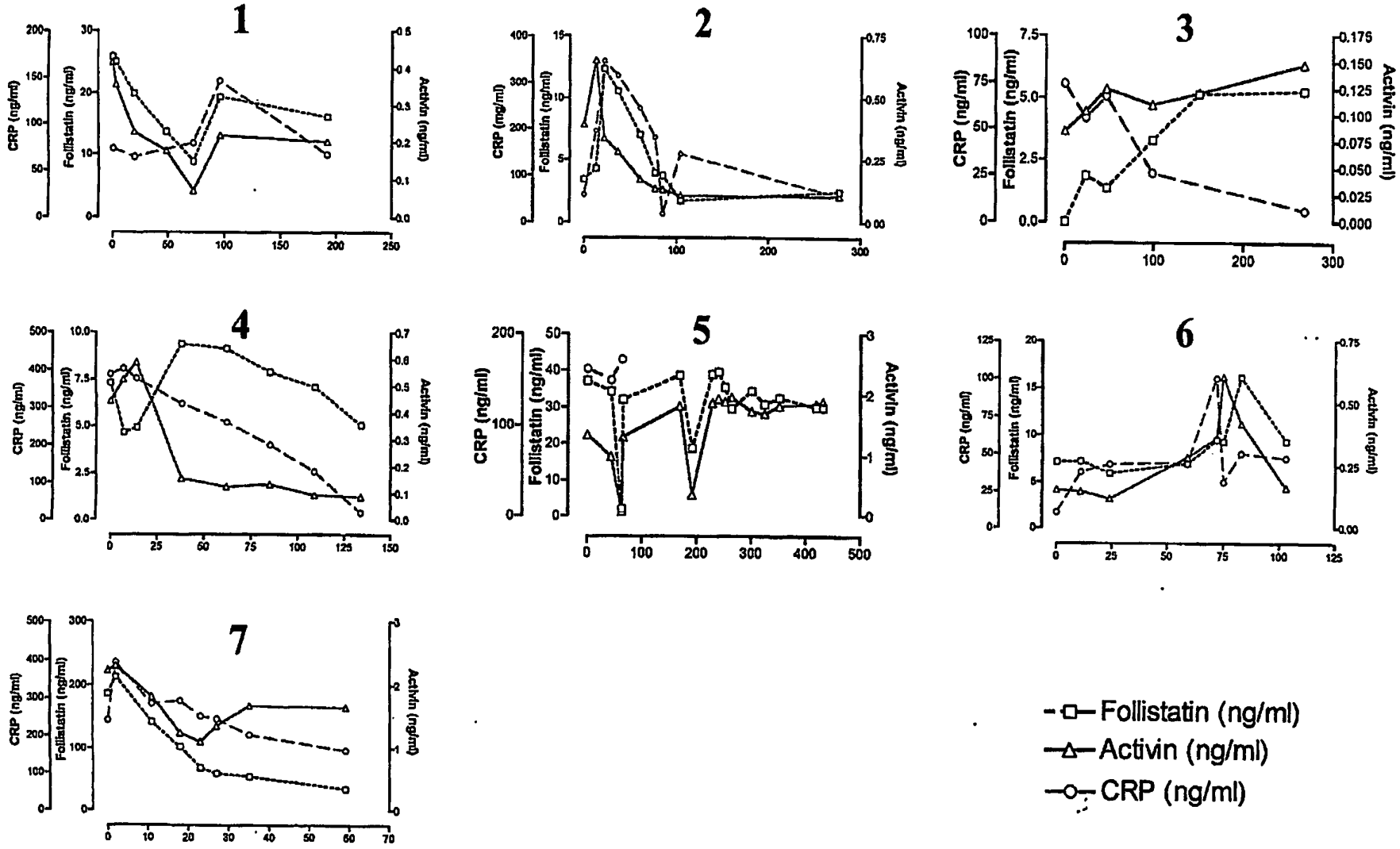
1
2
3
4
5
6
7
CRP (ng/ml)
Follistatin (ng/ml)
Activin (ng/ml)
Follistatin (ng/ml)
Activin (ng/ml)
CRP (ng/ml)
hours

FIG 1

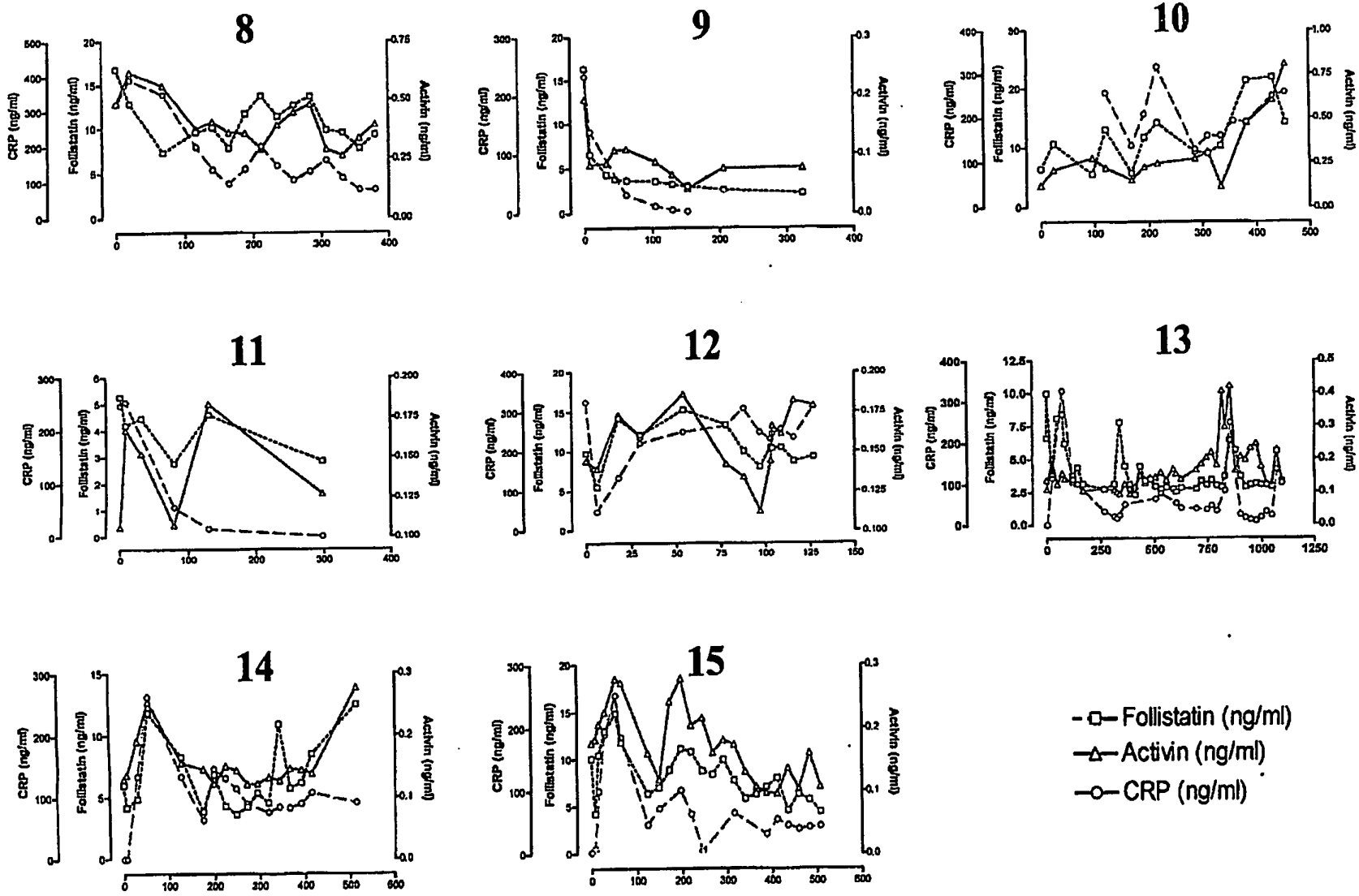

FIG 1 continued

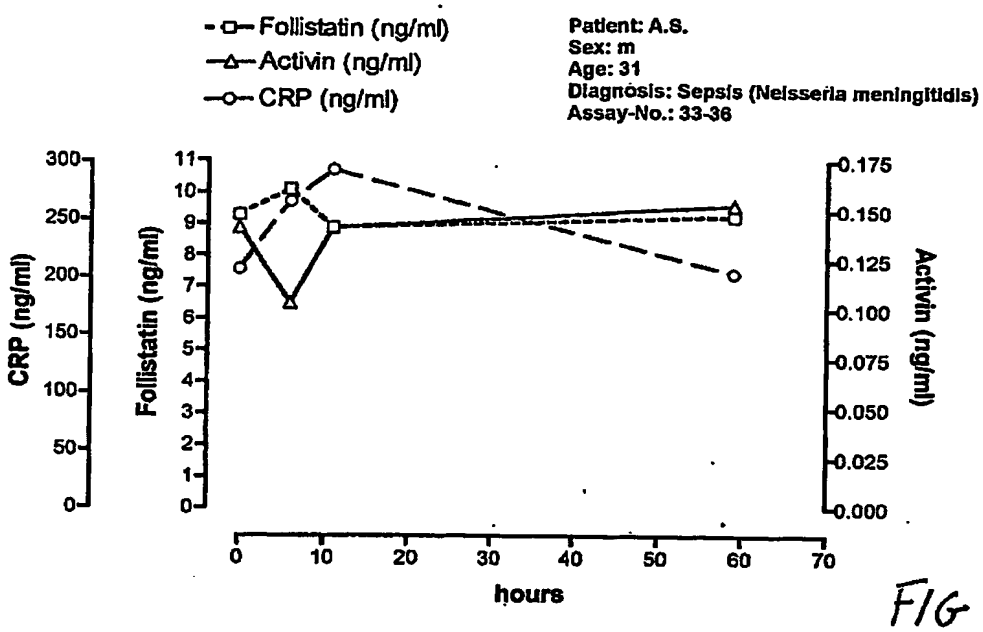
Follistatin (ng/ml)
Activin (ng/ml)
CRP (ng/ml)
Patient: A.S.
Sex: m
Age: 31
Diagnosis: Sepsis (Neisseria meningitidis)
Assay-No.: 33-36
CRP (ng/ml)
Follistatin (ng/ml)
Activin (ng/ml)
hours

FIG 2

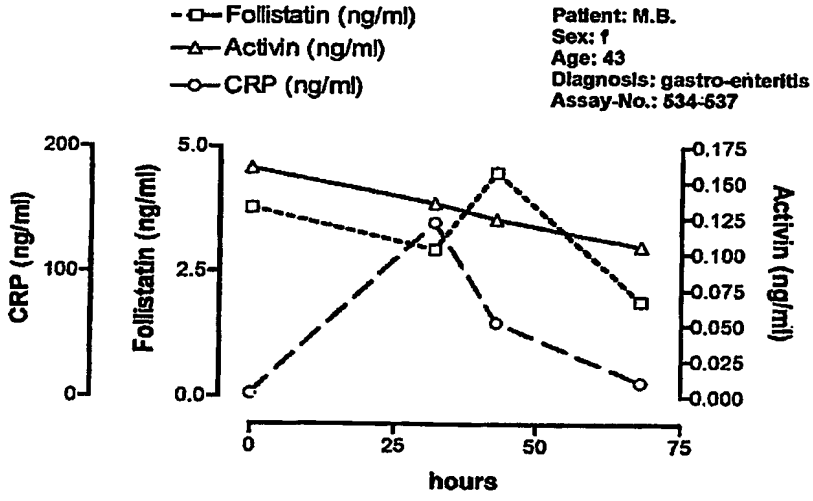
Follistatin (ng/ml)
Activin (ng/ml)
CRP (ng/ml)
Patient: M.B.
Sex: f
Age: 43
Diagnosis: gastro-enteritis
Assay-No.: 534-537
CRP (ng/ml)
Follistatin (ng/ml)
Activin (ng/ml)
hours

FIG 3

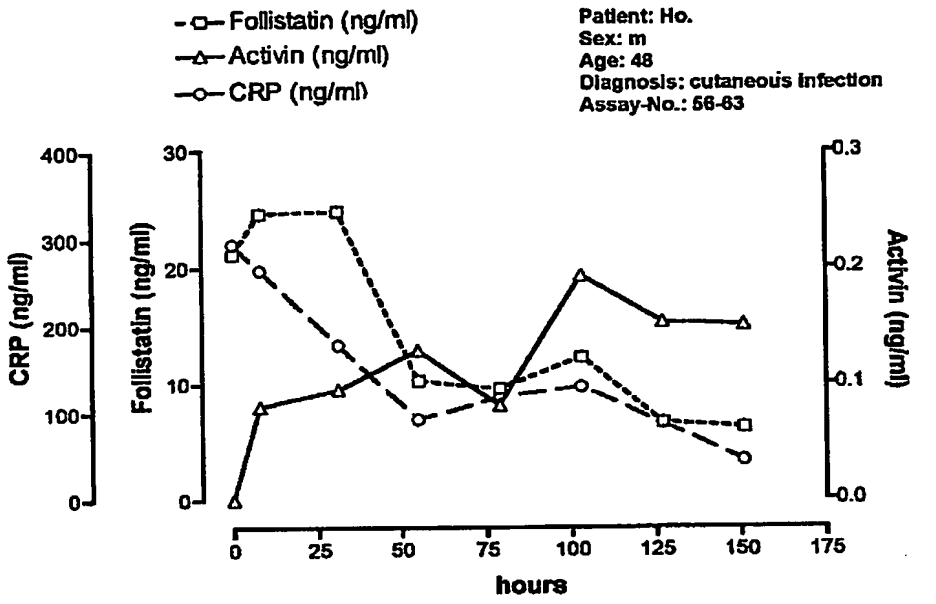
Follistatin (ng/ml)
Activin (ng/ml)
CRP (ng/ml)
Patient: Ho.
Sex: m
Age: 48
Diagnosis: cutaneous infection
Assay-No.: 56-63
CRP (ng/ml)
Follistatin (ng/ml)
Activin (ng/ml)
hours

FIG 4

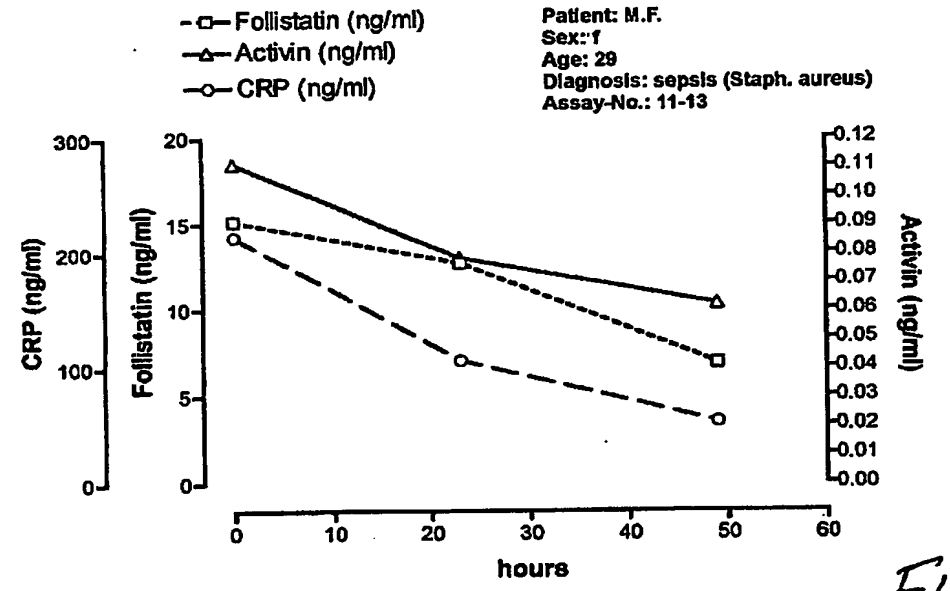
Follistatin (ng/ml)
Activin (ng/ml)
CRP (ng/ml)
Patient: M.F.
Sex: f
Age: 29
Diagnosis: sepsis (Staph. aureus)
Assay-No.: 11-13
CRP (ng/ml)
Follistatin (ng/ml)
Activin (ng/ml)
hours

FIG 5

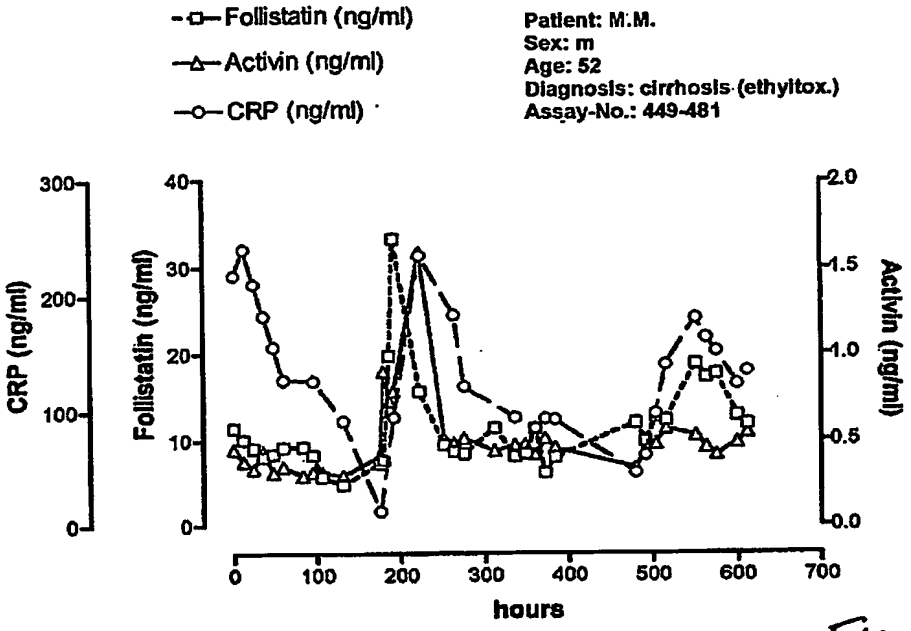
Follistatin (ng/ml)
Activin (ng/ml)
CRP (ng/ml)
Patient: M.M.
Sex: m
Age: 52
Diagnosis: cirrhosis (ethyltox.)
Assay-No.: 449-481
CRP (ng/ml)
Follistatin (ng/ml)
Activin (ng/ml)
hours

FIG 6

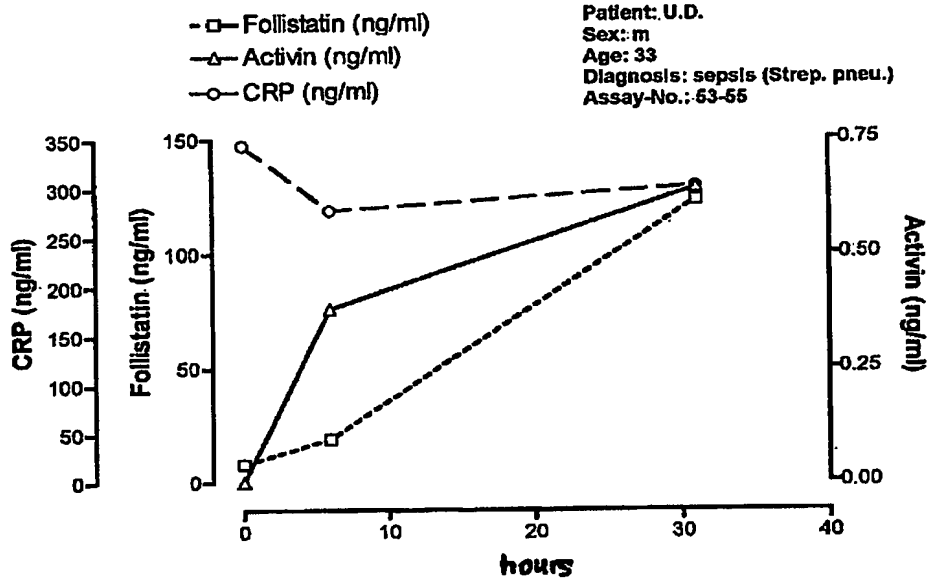
Follistatin (ng/ml)
Activin (ng/ml)
CRP (ng/ml)
Patient: U.D.
Sex: m
Age: 33
Diagnosis: sepsis (Strep. pneu.)
Assay-No.: 53-55
CRP (ng/ml)
Follistatin (ng/ml)
Activin (ng/ml)
hours

FIG 7

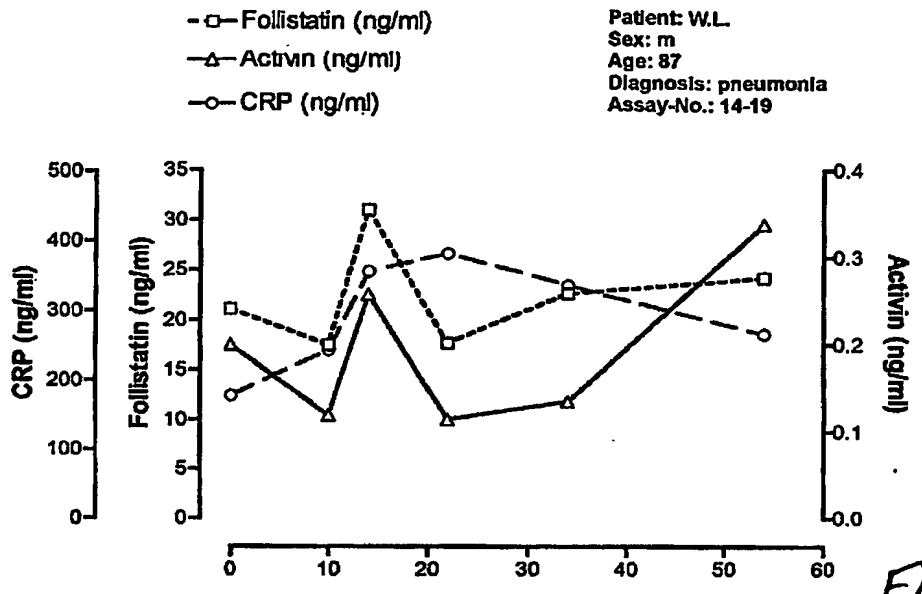
Follistatin (ng/ml)
Activin (ng/ml)
CRP (ng/ml)
Patient: W.L.
Sex: m
Age: 87
Diagnosis: pneumonia
Assay-No.: 14-19
CRP (ng/ml)
Follistatin (ng/ml)
Activin (ng/ml)

FIG 8

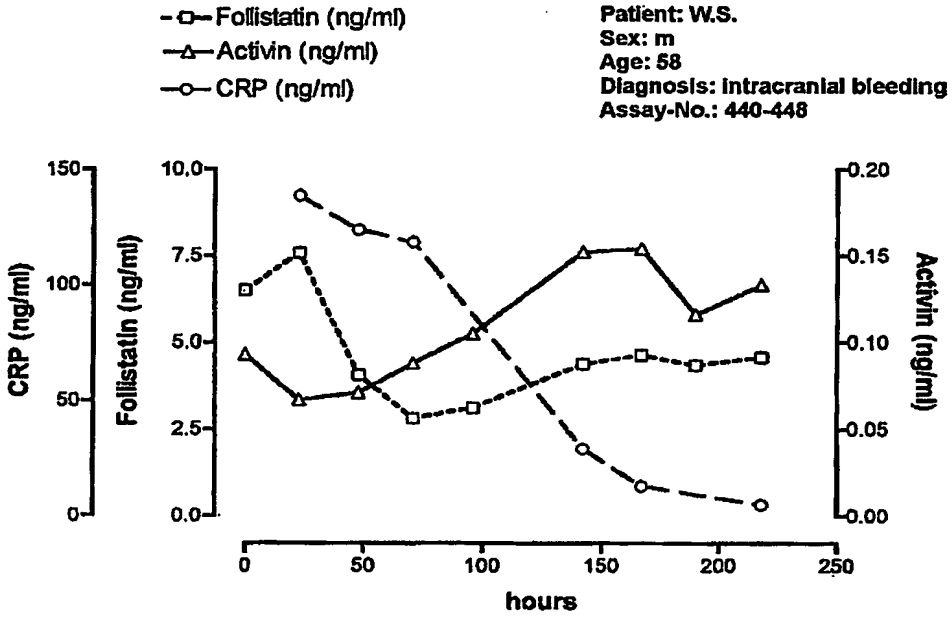
Follistatin (ng/ml)
Activin (ng/ml)
CRP (ng/ml)
Patient: W.S.
Sex: m
Age: 58
Diagnosis: intracranial bleeding
Assay-No.: 440-448
CRP (ng/ml)
Follistatin (ng/ml)
Activin (ng/ml)
hours

FIG 9

This Page is inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLORED OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REPERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning documents *will not* correct images problems checked, please do not report the problems to the IFW Image Problem Mailbox